

**WHAT IS CLAIMED IS:**

1. A method for the preparation of evolved microorganisms permitting a modification of metabolic pathways, characterized in that it comprises the following  
5 steps:
  - a) The preparation of a modified microorganism by genetic modification of cells of an initial microorganism so as to inhibit the production or consumption of a metabolite when that microorganism is grown on a defined medium, thereby impairing the ability of that microorganism to grow.
  - 10 b) The culture of the modified microorganism thereby obtained on said defined medium to cause it to evolve, where the defined medium can contain a co-substrate to allow such evolution.
    - a) The selection of a modified microorganism able to grow on said defined medium, if necessary with a co-substrate.
- 15 2. The method as claimed in Claim 1, characterized in that the metabolic pathway is chosen from among: biosynthesis pathways of amino acids, synthesis pathways of nucleic acids, synthesis pathways of lipids, or metabolism pathways of sugars.
3. The method as claimed in Claim 2, characterized in that the modified  
20 metabolic pathway is a biosynthesis pathway of amino acids.
4. The method as claimed in Claim 3, characterized in that the modified metabolic pathway is a biosynthesis pathway of an amino acid chosen from among: methionine, cysteine, threonine, lysine, or isoleucine.
5. The method as claimed in Claim 2, characterized in that the modified  
25 metabolic pathway consumes NADPH.
6. The method as claimed in one of claims 1 to 4, characterized in that the modification made in step a) favors the reduction of NADP to NADPH, possibly by limiting the oxidation of NADPH to NADP.
7. The method as claimed in Claim 1, characterized in that the evolved  
30 microorganism possesses at least one evolved gene coding for an evolved protein, the evolution of which replaces the inhibited metabolic pathway by a new metabolic pathway.

8. The method as claimed in one of Claims 1 or 2, characterized in that it includes an additional step a1), of introducing at least one heterologous gene coding for a heterologous protein, which heterologous gene is to allow the evolution of a new metabolic pathway, preparatory to step b) in which the modified  
5 microorganisms are cultured.

9. The method as claimed in one of Claims 7 or 8, characterized in that it includes a step d) of isolating the evolved gene coding for the evolved protein.

10. The method as claimed in Claim 9, characterized in that the evolved gene is introduced, in an appropriate form, into a production microorganism intended  
10 for the production of the evolved protein.

11. An evolved microorganism obtainable by a method according to any one of Claims 1 to 10

12. An evolved microorganism according to Claim 11, characterized in that the microorganism is the strain *E. coli* K183 with a modified "methionine  
15 synthase" activity, registered April 2, 2003 under the number I-3005 at the CNCM.

13. A method for the preparation of an evolved protein, wherein the evolved microorganism according to Claim 11 is cultivated in a culture medium appropriate for the production of the evolved protein.

14. The method as claimed in Claim 13, characterized in that the produced  
20 evolved protein is purified.

15. An evolved gene coding for an evolved protein obtainable by a method according to Claim 9.

16. An evolved protein obtainable by a method according to one of Claims 13 or 14.

25 17. An evolved protein according to Claim 16, characterized in that the enzyme has a modified "methionine synthase" activity and is chosen from cystathionine- $\gamma$ -synthases and acylhomoserine sulphydrylases with modified "methionine synthase" activity.

18. An evolved protein according to Claim 17, characterized in that the  
30 cystathionine- $\gamma$ -synthase with non-modified "methionine synthase" activity before the evolution is selected from cystathionine- $\gamma$ -synthases corresponding to PFAM with the reference PF01053 and COG with the reference CPG0626.

19. An evolved protein according to Claim 18, where the cystathionine- $\gamma$ -synthase with non-modified "methionine synthase" activity before the evolution, comprises the following amino acid sequence in its C-terminus (conserved region 1)

X1-X2-X3-L-G-X4-X5-X6-X7-X8-X9

5 In which

X1 represents A,G,S, preferentially A

X2 represents E,V,P,T, preferentially E

X3 represents S,T,N, preferentially S

X4 represents G,D,A,H,T, preferentially G

10 X5 represents V,A,T,H,N, preferentially V

X6 represents E,R,K,F, preferentially E

X7 represents S,T, preferentially S

X8 represents L,I,V,A, preferentially L et

X9 represents I,V,A,T, preferentially I.

15 Corresponding to residues 324 to 334 of the cystathionine- $\gamma$ -synthase sequence of *E. coli* K12, represented by SEQ ID NO 6.

20. An evolved protein according to Claims 18 or 19, characterized in that the cystathionine- $\gamma$ -synthase with non-modified "methionine synthase" activity before the evolution, comprises the following amino acid sequence in its N-terminus  
20 (conserved region 2)°

X10-X11-Y-X12-R-X13-X14-X15-X16-X17-X18

In which

X10 represents A, H, Y, F, L, K, preferentially A

25 X11 represents Y, E, D, K, R, V, I, preferentially Y

X12 represents S,A,T,P,G, preferentially S

X13 represents I,S,T,R,E,F,W,D, preferentially S

X14 represents S,G,A,I,E,N,K,P, preferentially G

X15 represents N,H,Q,S, preferentially N

30 X16 represents P,D,L, preferentially P

X17 represents T,M,N,G,S, preferentially T et

X18 represents R,L,V,S,W,E, preferentially R.

corresponding to residues 44 to 54 of the cystathionine- $\gamma$ -synthase sequence of *E. coli* K12, represented by SEQ ID NO 6.

21. An evolved protein according to any one of Claims 18 to 20 characterized in that it comprises at least one mutation in its C-terminal part and/or at  
5 least one mutation in its N-terminal part.

22. An evolved protein according to Claim 21, characterized in that the mutation consists in replacing an acidic amino acid, which interacts with the co-substrate cysteine in the non-modified enzyme, by a non-polar amino acid, selected from the residues glycine, alanine, leucine, isoleucine, valine, phenylalanine or  
10 methionine.

23. An evolved protein according to Claim 22, characterized in that the mutation in the C-terminal part of the cystathionine- $\gamma$ -synthase is introduced among the acidic amino acids of "conserved region 1" as defined in Claim 14, particularly into residue X2.

15 24. An evolved protein according to Claim 23, characterized in that it comprises the following amino acid sequence in its C-terminal part:

X1-X2-X3-L-G-X4-X5-X6-X7-X8-X9

In which

X1, X3, X4, X5, X6, X7, X8 et X9 are defined above and  
20 X2 represents G,A,L,I,V,F,M, preferentially A.

corresponding to residues 324 to 334 of the cystathionine- $\gamma$ -synthase sequence of *E. coli* K12, represented by SEQ ID NO 8.

25 25. An evolved protein according to Claim 24 characterized in that it comprises the following amino acid sequence in its C-terminal part:

A-A-S-L-G-G-V-E-S

corresponding to residues 324 to 332 of the cystathionine- $\gamma$ -synthase sequence of *E. coli* K12, represented by SEQ ID NO 8.

26. An evolved protein according to Claim 25 characterized in that the cystathionine- $\gamma$ -synthase with modified « methionine synthase » activity comprises  
30 the amino acid sequence represented by SEQ ID NO 8.

27. An evolved protein according to any of Claims 18 to 26 characterized in that the mutation in the N-terminal part of the cystathionine- $\gamma$ -synthase is introduced among the acidic amino acids of the conserved region 2, as defined above, in particular into residues X11 and/or R and/or X13.

5 28. An evolved protein according to Claim 27 characterized in that the cystathionine- $\gamma$ -synthase with modified « methionine synthase » activity comprises the following amino acid sequence in its N-terminal part:

X10-X11-Y-X12-X19-X13-X14-X15-X16-X17-X18

In which

10 X7, X9, X12, X14, X15, X16, X17 et X18 are defined above,

X11 is defined in Claim 15 or represents a non-polar amino acid,

X13 is defined in Claim 15 or represents a non-polar amino acid,

X19 is R or represents a non-polar amino acid, and

At least one of X11, X13 et X19 represents a non-polar amino acid,

15 where the non-polar amino acids are chosen independently among the residues glycine, alanine, leucine, isoleucine, valine, phenylalanine or methionine.

29. An evolved protein according to Claim 16, characterized in that the initial enzyme without mutations, before the evolution, catalyzes a sulfhydrylation reaction in the presence of H<sub>2</sub>S.

20 30. An evolved protein according to Claim 29, characterized in that the initial enzyme without mutations, before the evolution has O-acyl-L-homoserine sulfhydrylase activity.

31. An evolved protein according to Claim 30, characterized in that the initial enzyme with O-acyl-L-homoserine sulfhydrylase activity is chosen among the  
25 O-acyl-L-homoserine sulfhydrylases corresponding to PFAM reference PF01053 and COG reference COG2873.

32. An evolved protein according to Claim 31, characterized in that the initial enzyme is chosen among the following acylhomoserine sulfhydrylases:

NP\_785969 O-acetylhomoserine (thiol)-lyase, *Lactobacillus plantarum* WCFS1

30 AAN68137 O-acetylhomoserine sulfhydrylase, *Pseudomonas putida* KT2440

NP\_599886 O-acetylhomoserine sulfhydrylase, *Corynebacterium glutamicum*  
ATCC13032

NP\_712243 acetylhomoserine sulfhydrylase, *Leptospira interrogans* serovar lai str.  
56601

BAC46370 O-succinylhomoserine sulfhydrylase, *Bradyrhizobium japonicum*  
USDA110

5 AAO57279 O-succinylhomoserine sulfhydrylase, *Pseudomonas syringae* pv.  
tomato str. DC3000

NP\_284520 O-succinylhomoserine sulfhydrylase [*Neisseria meningitidis* Z2491

AAA83435 O-succinylhomoserine sulfhydrylase (*P. aeruginosa*)

33. An evolved protein according to Claim 31, characterized in that the  
10 initial enzyme before the evolution, is O-acyl-L-homoserine sulfhydrylase encoded  
by the *metY* gene of *Corynebacterium*.

34. An evolved protein according to Claim 33, characterized in that the O-  
acyl-L-homoserine sulfhydrylase is encoded by the *metY* gene of *Corynebacterium*  
*glutamicum* (Genbank AF220150).

15 35. The use of an evolved microorganism according to Claim 11 or an  
evolved protein according to Claim 15 in a biotransformation method.

36. A biotransformation method according to Claim 35 where the  
biotransformation is depending on NADPH dependant enzymes.

37. A method according to Claim 36 in which the NADPH-  
20 dependant enzymes have evolved substrate specificity.